



RESEARCH ARTICLE

ANTIFUNGAL ACTIVITY OF BIPHENYLS FROM *STREPTOMYCES* SP. BO07 AGAINST SELECTIVE PHYTOPATHOGENIC FUNGI OF RICE

^{1,*}Thongchai Taechowisan, ¹Suchanya Chaiseang and ²Waya S. Phutdhawong

¹Department of Microbiology, Faculty of Science, Silpakorn University, Thailand

²Department of Chemistry, Faculty of Science, Silpakorn University, Thailand

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ABSTRACT

The antifungal effect of two biphenyl compounds, 3'-hydroxy-5-methoxy-3,4-methylenedioxybiphenyl (1) and 3'-hydroxy-5,5'-dimethoxy-3,4-methylenedioxybiphenyl (2) against phytopathogenic fungi of rice; *Bipolaris oryzae*, *Fusarium semitectum*, *Fusarium fujikuroi* and *Curvularia lunata* was analyzed. Results of this study showed that compound 1 and 2 exhibited a very strong inhibitory activity against tested fungi with minimum inhibitory concentration (MIC) values of ≤ 128 $\mu\text{g/ml}$. Treatment with these compounds inhibited fungal radial growth, biomass formation, and spore density. The growth inhibition resulted from these compounds is due to the lysis of fungal cells indicated by the size of mycelia, in which the size of mycelia treated with these compounds is obviously smaller than that of control.

INTRODUCTION

Rice (*Oryza sativa* L.) is among the most important cereals in the world wide. Tropical and sub-tropical regions of the world are the major rice-producers, including Thailand. This important crop suffers from different microbial diseases, especially phytopathogenic fungi for example; rice brown leaf spot caused by *Bipolaris oryzae* Breda de Hann (formerly, *Helminthosporium oryzae*) (Teleomorph: *Cochliobolus miyabeanus*); a complex of plant diseases caused by *Fusarium semitectum* Berk. & Ravenel (syn: *F. pallidoroseum* (Cooke) Sacc.; *F. incarnatum* (Roberge) Sacc.); rice Bakanae disease caused by *Fusarium fujikuroi* (teleomorph: *Gibberella fujikuroi*); the *Curvularia* brown leaf spots and rice blight disease caused by *Curvularia lunata* (Wakker) Boed. These diseases are important rice diseases in the world (Padmanabhan, 1949; Padwick, 1950; Rangaswami, 1975; Neergaard, 1977; Ou, 1985; Phillips et al., 1992). They can be the serious diseases causing a considerable yield loss. Where, they affect the quality and the number of grains per panicle and reduce the kernel weight (Padmanabhan, 1965; Neergaard, 1981; Nyvall et al., 1995; Mew and Gonzales, 2002). In continuation of our search for biologically active constituents from endophytic actinomycetes. We have recently isolated *Streptomyces* sp. BO07 from the root tissue of *Boesenbergia*

rotunda (L.) Mansf A., the major active ingredients from the culture were identified as 3'-hydroxy-5-methoxy-3,4-methylene dioxybiphenyl (1) and 3'-hydroxy-5,5'-dimethoxy-3,4-methylenedioxybiphenyl (2), which displayed strong antibacterial activity against Gram-positive bacteria, were scavengers of the reactive oxygen species and had strong anticancer activity (Taechowisan et al., 2017). Since biphenyl compounds have been reported as antifungal agents (Kokubun et al., 1995; Johann et al., 2010; Rikhi et al., 2016). In the present work, we report these biphenyls exhibit potent antifungal activity against *Bipolaris oryzae*, *Fusarium semitectum*, *Fusarium fujikuroi* and *Curvularia lunata*, the important cause of rice diseases in Thailand.

MATERIALS AND METHODS

Extraction and Isolation

Streptomyces sp. BO-07 was isolated from the root tissue of *Boesenbergia rotunda* (L.) Mansf A. by the surface-sterilization technique and identified as described in our previous study (Taechowisan et al., 2003). Strain BO-07 was grown on ISP-2 agar at 30 °C for 14 days and then the culture medium was cut into small pieces that were extracted with ethyl acetate (3 x 500 ml). This organic solvent was pooled and then taken to dryness under rotary evaporation to give a dark brown solid (1.68 g). The solid was separated by column chromatography using silica gel 60 (Merck, 0.040-0.063 mm)

*Corresponding author: Thongchai Taechowisan,

Department of Microbiology, Faculty of Science, Silpakorn University, Thailand.

and petroleum/ethyl acetate (2:1 and 1:1) as the eluent to give five main fractions (F1-F5). Only fraction F3 (0.54 g) displayed antibacterial activity by disk diffusion method. This fraction was further separated by TLC (Merck, Si gel 60, 0.5 mm; hexane/ethyl acetate (3:2) to give 157 mg and 96 mg of pure compounds **1** and **2**, respectively. The structural determination of the compounds was made by spectral analyses as described in the previous report (Taechowisan et al., 2017).

Antifungal activity assay

The inhibitory effect of the compounds and the crude extract on phytopathogenic fungi was studied. The four fungi used in the antifungal assay were: *Bipolaris oryzae*, *Fusarium semitectum*, *Fusarium fujikuroi* and *Curvularia lunata*. The fungi were obtained from Mrs. Duangkamon Boonchuay, (Agricultural technical officer, Professional Level), Rice Department, Chai Nat Rice Research Center, Thailand and were subcultured on Potato Dextrose Agar (PDA) (Himedia, India) and incubated at $30 \pm 2^\circ\text{C}$ for 7 days.

Minimum inhibitory concentration test

The fungi were grown on Potato Dextrose agar (PDA) at $30 \pm 2^\circ\text{C}$ for 7 days to produce conidia. The fungal colonies were covered with 5 ml of sterile distilled water, and the suspensions were made by gently using fine brush. Inoculum quantification was made by counting the conidia in a hemacytometer which ranged from 2×10^6 to 4×10^6 CFU/ml. The inoculum suspensions were diluted in Potato Dextrose broth (PDB) medium to obtain a cell number ranging from 2×10^4 to 4×10^4 CFU. The microdilution plates (96 wells) were set up in accordance with the NCCLS reference method (2002). Each microdilution well containing 100 μl of the twofold compound or crude extract concentration (ranged from 2 to 1024 $\mu\text{g/ml}$) was inoculated with 100 μl of the diluted inoculum suspension. For each test plate, compound-free controls were included, one with the medium alone (sterile control) and the other with 100 μl of medium plus 100 μl of inoculum suspension (growth control). The microdilution plates were incubated at $30 \pm 2^\circ\text{C}$ and were read visually after 7 days of incubation. Reading and interpretation of MICs. Endpoint determination readings were performed visually based on comparison of the growth in wells containing the compounds or crude extract with that of the growth control. Carbendazim was used as standard control. MIC was defined as the lowest concentration showing 100% growth inhibition.

Test for inhibitory activity against fungal radial growth

Different concentrations of the compounds and the crude extract ($\mu\text{g/ml}$): 32, 128, and 512 were tested for antifungal activity on radial growth of the fungi on PDA medium. The compound or the crude extract was put in the center of Petri dish and then added with 10 ml melted PDA medium. The volume of the compound or the crude extract that was added into Petri dish was adjusted according to the concentration tested. Three Petri dishes were prepared for each concentration. The Petri dishes were shaken gently to allow the compounds and the extract to distribute evenly in PDA medium. Carbendazim (0.1 mg/ml) was used as standard control. Plates without the compounds and the extract stood as negative control. A mycelia plug (5 mm diam.) of the fungi taken from the edge of a 7-day old culture was put in the center

of PDA. The cultures were incubated at $30 \pm 2^\circ\text{C}$ for 7 days. The diameter of fungal colony was measured and recorded at 8th day of inoculation and incubation. The inhibitory activity to the radial growth was determined according to the following formula:

$$\text{IR (\%)} = \frac{\text{DC-DT}}{\text{DC}} \times 100$$

IR = inhibitory activity against radial growth in percent

DC = diameter of fungal colony without compounds or extract treatment (control)

DT = diameter of fungal colony treated with compounds or extract

Test for inhibitory activity against biomass formation

Potato dextrose broth (PDB) medium (approx. 45 ml each) was placed in 100-ml Erlenmeyer flasks and various concentrations of the compounds or the crude extract ($\mu\text{g/ml}$): 32, 128, and 512 were added into the flasks. The PDB medium was inoculated with spore's suspension of the fungi (10^5 spores/ml). The final volume of culture was adjusted to 50 ml by adding PDB medium. Three flasks were prepared for each concentration. The cultures were incubated at room temperature ($30 \pm 2^\circ\text{C}$) for 7 days. The biomass was harvested through centrifugation at 5,000 rpm for 10 minutes. The biomass was taken and placed on No.1 Whatman filter paper and dried in an oven at 60°C until constant weight. The inhibitory activity to the biomass formation was calculated according to the following formula:

$$\text{IB (\%)} = \frac{\text{WC-WT}}{\text{WC}} \times 100$$

IB = inhibitory activity to the fungal biomass in percent

WC = dry weight of biomass on control

WT = dry weight of biomass treated with compounds or extract

Test for inhibitory activity against spore's formation

Spores of the fungi were harvested from cultures maintained on PDA plates using fine brush and sterile distilled water. The PDB medium (10 ml) was put into test tubes with various concentrations of the compounds or the crude extract ($\mu\text{g/ml}$): 32, 128, and 512. Three tubes were prepared for each concentration. The cultures were incubated at room temperature ($30 \pm 2^\circ\text{C}$) for 7 days. The number of spores was counted using hemocytometer under light microscope. The inhibitory activity on spore's formation was calculated according to the following formula:

$$\text{IS (\%)} = \frac{\text{SDC-SDT}}{\text{SDC}} \times 100$$

IS = inhibitory activity against spore's formation

SDC = spore's density on control

SDT = spore's density with compounds or extract treatment

Morphological observation under light microscope

A 100 μl spore suspension of tested fungi (2.5×10^6 spores/ml) was mixed with melted PDA on a Petri dish. Four diffusion wells side by side were made on PDA medium using cork borer (5 mm diameter). Into respective well, 20 μl of the compounds or the crude extract (0, 32, 128, and 512 $\mu\text{g/ml}$) was added.

The cultures were incubated at room temperature (30 ± 2 °C) for 7 days. The colonial plugs were taken from the edge of inhibition zone using cork borer to be used as specimens. The specimens were observed and photographed under light microscopy with an immersion lens.

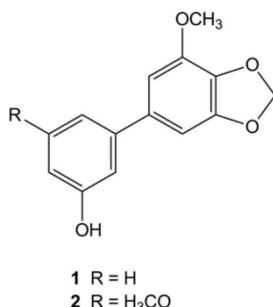


Figure 1. Chemical structures of 3'-hydroxy-5-methoxy-3,4-methylenedioxybiphenyl (1) and 3'-hydroxy-5,5'-dimethoxy-3,4-methylenedioxybiphenyl (2)

RESULTS AND DISCUSSION

Compounds 1 and 2 (Figure 1) showed antifungal activity at concentrations ≤ 128 $\mu\text{g/ml}$ against four phytopathogenic fungi of rice (Table 1). Compound 2 displayed activity against all the test fungi with MIC values varying from 32 to 128 $\mu\text{g/ml}$. The crude extract was active less than these compounds with the highest concentration tested (256 to 512 $\mu\text{g/ml}$).

Table 1. Minimum inhibitory concentrations ($\mu\text{g/ml}$) of the crude extract and isolated compounds on phytopathogenic fungi

Test agents	Phytopathogenic fungi			
	B.o. ^a	F.s.	F.f.	C.I.
Crude extract	256	512	512	256
Compound 1	64	128	128	128
Compound 2	32	64	64	128
Carbendazim	16	32	32	16

^aB.o.; *Bipolaris oryzae*, F.s.; *Fusarium semitectum*, F.f.; *Fusarium fujikuroi* and C.I.; *Curvularia lunata*

The most active compound against these fungi was compound 2, substitution at 5'-C with methoxy group was more active. According to Gutiérrez *et al.* have shown that tetrahydroquinolines with a methoxy group showed interesting activity against *Cladosporium cladosporioides* with a MIC value of 13.75 $\mu\text{g/ml}$ (Gutiérrez *et al.*, 2012). Chávez *et al.* reported that the inhibitory effects on the mycelium growth of plant pathogen *Botrytis cinerea* by geranylphenols depended on the substitution of one, two and three methoxy groups in the aromatic ring, it was increased from 40% to 90% by increasing the number of methoxy groups (Chávez *et al.*, 2015). Caruso *et al.* reported that the presence of methoxy groups improved the antifungal activity of stilbene derivatives against *B. cinerea* (Caruso *et al.*, 2011). A positive correlation among antifungal activity of natural and synthetic stilbenes and their hydrophobicity was found, suggesting that pterostilbene (has two methoxy groups) is more active than the less hydrophobic resveratrol (no methoxy group), due to its increased diffusion through the cytoplasmic membrane (van Baarlen *et al.*, 2004). Methoxylation is an important molecular feature for membrane penetration, this feature may contribute to the antifungal activity. In addition, we found that these compounds inhibited the diameter of fungal colony, biomass formation and spore density as presented in Table 2, Table 3 and Table 4. Treatment with compound 1 and 2 at concentration of 32 $\mu\text{g/ml}$ inhibited the fungal growth by 52.8% to 63.2% and 61.4% to 76.3% respectively. Similar inhibitory activity was shown against biomass formation, where treatment with compound 1 and 2 at concentration of 32 $\mu\text{g/ml}$ inhibited biomass formation by 62.8% to 77.6% and 75.8% to 82.5% respectively, where treatment with compound 1 and 2 at the same concentration inhibited spore density by 35.1% to 47.7% and 46.4% to 56.1% respectively. The completed inhibitory activity of compound 1 and 2 against the diameter of fungal colony, biomass formation and spore density occurred on treated cultures of all the tested fungi was at concentration of 512 $\mu\text{g/ml}$.

Table 2. Inhibitory activity of the crude extract and isolated compounds against diameter of colony of phytopathogenic fungi

Test agents	Concentration ($\mu\text{g/ml}$)	Diameter of colony (mm) of tested fungi			
		B.o. ^a	F.s.	F.f.	C.I.
None (control)	-	77.2	59.7	66.0	75.9
Crude extract	128	44.5(42.3%)	37.3(37.5%)	43.9(33.4%)	45.1(40.6%)
	512	NG ^b (100%)	NG (100%)	NG (100%)	NG (100%)
Compound 1	32	28.4(63.2%)	28.1(52.8%)	24.7(55.7%)	24.3(58.4%)
	128	7.7(90.0%)	9.4(84.3%)	9.2(86.1%)	8.2(89.2%)
	512	NG (100%)	NG (100%)	NG (100%)	NG (100%)
Compound 2	32	22.0(71.5%)	21.1(64.7%)	25.5(61.4%)	18.0(76.3%)
	128	2.9(96.2%)	1.2(98.0%)	2.9(95.5%)	0.7(99.1%)
	512	NG (100%)	NG (100%)	NG (100%)	NG (100%)
Carbendazim	32	NG (100%)	NG (100%)	NG (100%)	NG (100%)

^aB.o.; *Bipolaris oryzae*, F.s.; *Fusarium semitectum*, F.f.; *Fusarium fujikuroi* and C.I.; *Curvularia lunata*. ^bNG; no growth. +

Table 3. Inhibitory activity of the crude extract and isolated compounds against biomass formation of phytopathogenic fungi

Test agents	Concentration ($\mu\text{g/ml}$)	Biomass (g/50 ml) of tested fungi			
		B.o. ^a	F.s.	F.f.	C.I.
None (control)	-	0.263	0.104	0.193	0.115
Crude extract	128	0.128(51.1%)	0.053(48.6%)	0.107(44.2%)	0.053(53.5%)
	512	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Compound 1	32	0.059(77.6%)	0.039(62.8%)	0.050(74.1%)	0.039(66.3%)
	128	0.006(97.7%)	0.008(92.3%)	0.007(96.4%)	0.011(90.4%)
	512	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Compound 2	32	0.063(75.8%)	0.023(77.9%)	0.033(82.5%)	0.023(79.5%)
	128	0.005(98.1%)	0.003(97.1%)	0.001(99.5%)	0.001(99.1%)
	512	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Carbendazim	32	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)

^aB.o.; *Bipolaris oryzae*, F.s.; *Fusarium semitectum*, F.f.; *Fusarium fujikuroi* and C.I.; *Curvularia lunata*.

The structural observation of mycelia of the tested fungi under light microscope showed that there was a difference of the surface and size of the mycelia of the fungi with out and with treatment of the compounds or crude extract as shown in Figure 2.

The size of mycelia with compounds or crude extract treatment was obviously smaller than that of mycelia without treatment (control). This condition is probably due to the lysis of mycelia cells caused by these compounds. Observation under light microscope showed that the size of hyphae and spore were

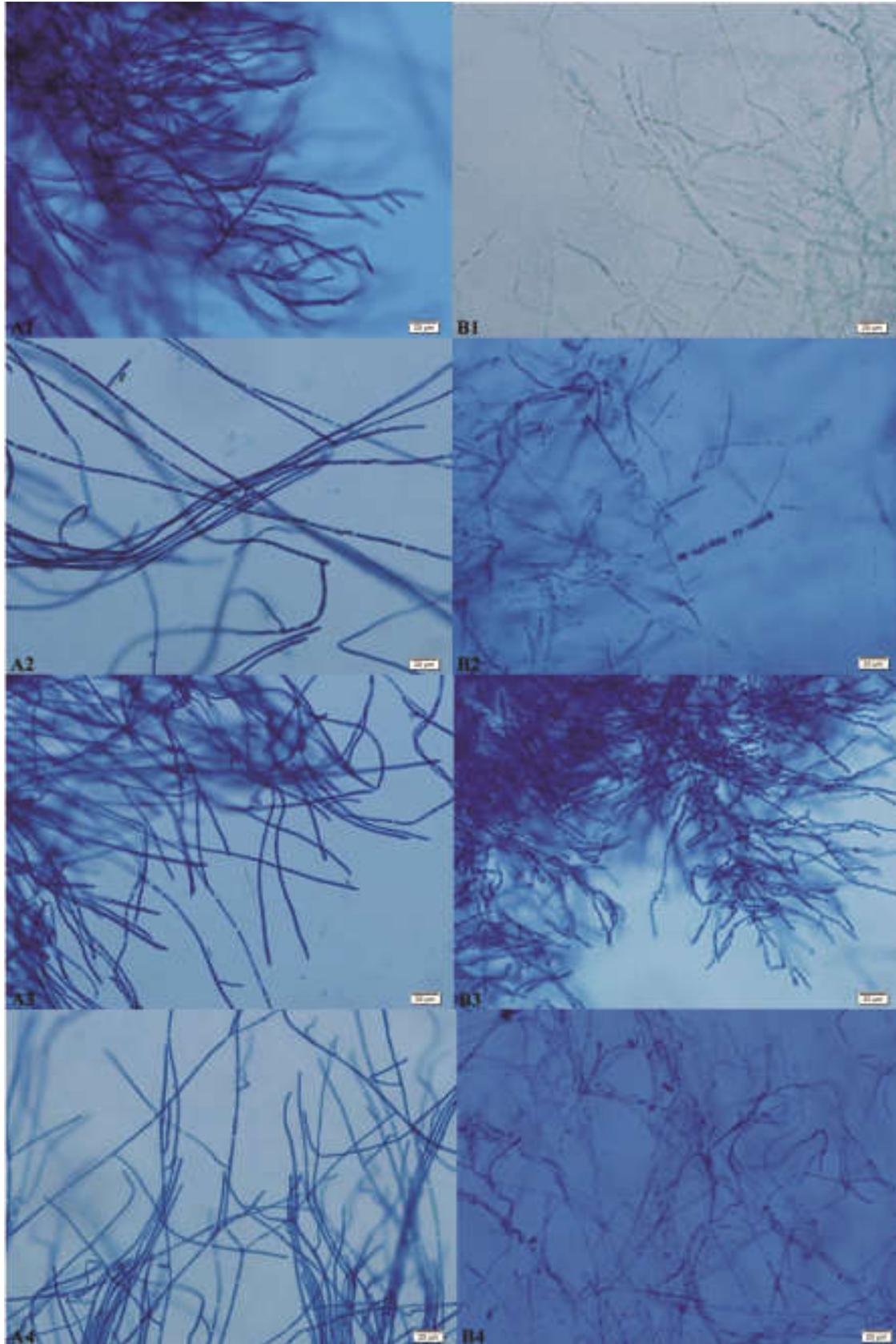


Figure 2. Light microscopic images of fungal mycelium (1: *Bipolaris oryzae*; 2: *Fusarium semitectum*; 3: *Fusarium fujikuroi*; 4: *Curvularia lunata*) treated with and without the compounds after incubation at $30 \pm 2^\circ\text{C}$ for 7 days. (A): mycelia showed normal size after treated without the compounds; (B): mycelia lysis after treated with $512 \mu\text{g/ml}$ of compound 2. Bar = $20 \mu\text{m}$

Table 4. Inhibitory activity of the crude extract and isolated compounds against spore density of phytopathogenic fungi

Test agents	Concentration ($\mu\text{g/ml}$)	Spore density (spore/ml $\times 10^4$) of tested fungi			
		B.o. ^a	F.s.	F.f.	C.l.
None (control)	-	23.7	31.9	22.6	24.5
Crude extract	128	17.2(27.4%)	22.4(29.8%)	16.3(27.9%)	18.2(25.7%)
	512	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Compound 1	32	15.4(35.1%)	16.7(47.7%)	14.1(37.8%)	13.6(44.6%)
	128	0.4(98.3%)	0.3(99.0%)	0.5(97.8%)	0.7(97.1%)
	512	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Compound 2	32	12.1(48.9%)	14.0(56.1%)	10.2(54.9%)	13.1(46.4%)
	128	0.2(99.2%)	0.1(99.7%)	0.2(99.1%)	0.4(98.4%)
	512	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)
Carbendazim	32	0.0 (100%)	0.0 (100%)	0.0 (100%)	0.0 (100%)

^aB.o.; *Bipolaris oryzae*, F.s.; *Fusarium semitectum*, F.f.; *Fusarium fujikuroi* and C.l.; *Curvularia lunata*.

smaller when compared to control because of lysis of the cells. Several mode of actions may happened resulted from antifungal substances such as the alteration of cell permeability, degradation of cell wall, inhibition of enzymatic activities in the fungal cells that in turn affected the cell membrane permeability (Semangun, 2006). This phenomenon occurred on the crude extract that contain phenyl compounds. These substance actively precipitated the protein which resulted in disintegration of fungal cell membrane. The surface tension of cell membrane is reduced and resulted in cell lysis. As a consequence, the growth of a fungus is suppressed (Rachmawati, 2009). The inhibitory activity of the compounds against the diameter of colony, biomass formation and spore density of these phytopathogenic fungi was significantly increased in a concentration-dependent manner. For These biphenyl compounds, the antifungal activity increases by increasing the number of methoxy groups in the aromatic ring. The antifungal activity does not only affect on MIC values but it also affect on the mycelial growth (radial growth and biomass formation) and sporulation. Present study revealed that biphenyl compound 1 and 2 showed a remarkable antifungal activity against phytopathogenic fungi through lysis of mycelial cells, suggested that these compounds potentially can be used as an alternative agent to control rice diseases. The field research is needed to evaluate the effectiveness of these compounds to control rice diseases.

Conclusion

This work shows the antifungal activity of biphenyl compounds; 3'-hydroxy-5-methoxy-3,4-methylene dioxybiphenyl (**1**) and 3'-hydroxy-5,5'-dimethoxy-3,4-methylenedioxybiphenyl (**2**), isolated from the culture of *Streptomyces* sp. BO07; an endophyte in *Boesenbergia rotunda* (L.) Mansf A. Compound 2, with the 2 electron-donating methoxy groups, can be evaluated in these assays to study a possible mechanism of action. Structure-activity relationship indicated the relevance of the number of methoxy group was more active than the other compound. These results can be used for the development of new compounds for the treatment of fungal diseases in agriculture. Since these biphenyl compounds have antagonism against phytopathogenic fungi of rice. They should be further studied on fungicidal actions in field trial or on whole plant method in a greenhouse. The other biological activities of these biphenyl compounds should be studied as well.

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